Induction of Hyperplasia and Increased DNA Content in the Uterus of Immature Rats Exposed to Coumestrol

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Administration of the phytoestrogen coumestrol to ovariectomized rats leads to increases in both wet and dry uterine weights in the absence of an increase in uterine DNA content, as reported by Markaverich et al. [Effects of Coumestrol on Estrogen Receptor Function and Uterine Growth in Ovariectomized Rats. Environ Health Perspect 103:574–581 (1995)]. It was not possible to know if the observed atypical uterotrophic response of coumestrol was associated uniquely with the ovariectomized uterotrophic assay protocol. This question is answered in the present paper. Two experiments are described in which three daily oral gavage administrations of 60 mg/kg/day coumestrol to immature AP rats were followed by assessment of the reproductive tract on the fourth day. In both experiments coumestrol increased uterine fluid content and increased the weights of the uterus, cervix, and vagina. In addition, bromodeoxyuridine staining of uterine sections enabled confirmation of uterine hyperplasia for the coumestrol-treated animals. In the second experiment, total uterine DNA was determined; it doubled in the coumestrol-treated animals. Estradiol benzoate acted as the positive control agent for both of these experiments, and in each case it gave similar responses to those seen for coumestrol. We conclude that the uterotrophic activity of the phytoestrogen coumestrol in the immature intact rat is typical of the activity of the natural estrogen estradiol. *Key words:* coumestrol, DNA synthesis, hyperplasia, immature, rodent, ovariectomized, uterotrophic. *Environ Health Perspect* 107:819–822 (1999). [Online 3 September 1999]
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Markaverich et al. (*1*) reported that administration of the phytoestrogen coumestrol (COUM) to ovariectomized rats, either as a single subcutaneous injection or via drinking water for 5 days, leads to increases in both wet and dry uterine weights. However, these tissue weight increases were not considered to be caused by cellular hyperplasia because there was no commensurate increase in DNA content of the uteri. These results were in contrast to those seen by other investigators for COUM when using the immature intact rat uterotrophic assay (*2,3*), although Markaverich et al. (*1*) noted that whether or not COUM induces cellular hyperplasia and increases in DNA content in those earlier studies was not evaluated.

Given the increasing reliance on the rodent uterotrophic assay as a primary measure of the estrogenic activity of chemicals to rodents, the prospect that some agents that are active in this assay may not have caused uterine hyperplasia is worthy of further study. Because we recently confirmed the activity of COUM in the immature rat uterotrophic assay (*4*), we repeated and extended those experiments to include an assessment of uterine hyperplasia (bromodeoxyuridine staining) and total uterine DNA content. In addition, the surgical procedure was modified to enable accurate determination of uterine luminal fluid content and to enable monitoring of possible changes to the weight of the cervix and vagina. The presumed involvement of the estrogen receptor (ER) in the uterotrophic activity of COUM was evaluated by conducting experiments in the presence and absence of the ER blocking agent Faslodex (FAS; ICI 182,780; Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK) (*5*). Estradiol benzoate (E_2B) was used as a positive control for these experiments.

Materials and Methods

Chemicals. COUM, E_2B, FAS, and arachis oil (AO) were used as previously described (*4*). 5-Bromodeoxyuridine (BrdU), 3,3′-diaminobenzidine, orcinol, and calf thymus DNA were obtained from Sigma Chemical Company (Poole, Dorset, UK). All antibodies were supplied by DAKO Ltd. (High Wycombe, UK). Yeast RNA was purchased from Calbiochem (Nottingham, UK).

Animals. Immature female Alpk:AP rats (21–22 days old) with body weights in the range of 38–48 g were obtained from the breeding unit at Zeneca, Alderley Park (Macclesfield, Cheshire, UK). Animals were housed in wire mesh cages with solid bottoms. Humidity and temperature were controlled and a 12-hr/12-hr light–dark cycle was maintained. Animals were weaned on R&M No. 3 diet at 19–21 days old and maintained on R&M No. 1 diet from 21 days onward (both diets were obtained from Special Diet Services Ltd., Witham, Essex, UK). Diet and water were available *ad libitum.* All animals were acclimatized for 24 hr before being dosed.

Uterotrophic assays. The uterotrophic activities of COUM, E_2B, and FAS were evaluated using the test protocol described by Odum et al. (*4*). The test agents were dissolved (E_2B) or homogeneously suspended (*4*) (COUM and FAS) in AO and dosed by oral gavage on 3 successive days. Each animal received either two doses (one immediately following the other) of the appropriate compound combination/day (experiment 1, Table 1) or a single dose of the appropriate compound (experiment 2, Table 1) using a dosing volume of 5 ml/kg body weight. AO-dosed animals acted as vehicle controls. Animals were sacrificed using an overdose of Fluothane (Zeneca Pharmaceuticals) followed by cervical dislocation 24 hr after the final dose. Vaginal opening (or otherwise) was recorded at the time of death. Test groups, daily dose levels, and animal group sizes are shown in Table 1. In the first experiment BrdU was administered in the drinking water [0.8 mg/mL, based on the method of Carthew et al. (*6*)] for the duration of the experiment. The BrdU solution was contained in glass drinking bottles covered in aluminum foil to reduce photodecomposition of the BrdU.

The reproductive tract was excised from the junction of the uterine horns with the ovaries to the external involution of the vagina, trimmed free of fat, and weighed without puncturing the uterus. The vagina was removed and weighed. The cervix was removed and discarded. The remaining uterus (intact uterus) was weighed again prior to being punctured, blotted to remove excess water, and reweighed [uterine wet weight; (*4*) and as described here]. The uterus was then cut such that the right horn could be dried at 70°C for 24 hr (*4*) and reweighed to obtain a dry weight (adjusting the uterine weight to give total uterine dry weight). The left horn of the uterus, together with the junction to the cervix, was processed for either BrdU staining (experiment 1) or total DNA content determination (experiment 2). From these measurements the uterine luminal fluid was also calculated. Cervical weight was determined by default based on the recorded weight of the entire reproductive tract minus the recorded weights of both the vagina and the intact uterus (prior to blotting).

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Table 1. Activity of E_2B, COUM, and FAS in the immature rat uterotrophic assay.

Experiment no., daily doses	Uterus (mg) Intact Ind	Intact Mean ± SD	Wet (blotted) Ind	Wet (blotted) Mean ± SD	Dry Ind	Dry Mean ± SD	Vagina (mg) wet (blotted) Ind	Vagina (mg) wet (blotted) Mean ± SD	Cervix (mg) wet (calculated) Ind	Cervix (mg) wet (calculated) Mean ± SD	Total ULF (mg) (calculated) Ind	Total ULF (mg) (calculated) Mean ± SD	DNA/uterus (µg) Ind	DNA/uterus (µg) Mean ± SD
Experiment 1														
AO (5 mL/kg × 2)	25.2	21.6 ± 2.6	23.3	18.8 ± 2.6	5.4	4.7 ± 0.9	32.6	27.0 ± 3.3	7.8	7.4 ± 1.0	19.8	17.0 ± 2.6	ND	ND
	18.1		16.5		4.1		27.4		9.0		14.0			
	24.4		16.0		3.5		25.4		0.1[a]		20.9			
	21.1		19.2		5.6		28.5		7.3		15.5			
	20.5		17.3		3.7		25.2		6.5		16.8			
	19.4		18.3		4.9		22.2		7.8		14.5			
	22.6		20.8		5.4		27.8		6.2		17.2			
E_2B (400 µg/kg) + AO (5 mL/kg)	94.5	133.9 ± 48.9 **,#	91.1	99.6 ± 19.4 **,##	17.9	18.7 ± 3.5 **,##	47.6	54.9 ± 7.4 **,#	15.2	20.0 ± 4.2 **	76.6	115.2 ± 45.6	ND	ND
	117.9		96.5		16.7		56.4		26.2		101.2			
	85.0		81.0		16.5		63.0		17.4		68.5			
	230.3		139.1		26.0		43.9		25.2		204.3			
	148.9		104.9		19.1		64.1		19.1		129.8			
	147.1		100.8		18.8		53.8		19.8		128.3			
	113.3		84.1		15.7		55.2		17.3		97.6			
COUM (60 mg/kg) + AO (5 mL/kg)	87.5	83.9 ± 5.0 **	71.8	73.7 ± 4.8 **	14.1	13.9 ± 1.0 **	65.0	67.3 ± 9.5 **	19.7	19.8 ± 4.3 **	73.4	70.0 ± 4.35	ND	ND
	92.1		83.3		15.8		77.9		19.1		76.4			
	82.7		71.5		13.3		76.7		28.7		69.4			
	78.9		74.9		14.4		71.1		17.9		64.5			
	82.7		70.6		14.1		66.5		14.5		68.6			
	77.7		68.9		12.5		64.1		18.9		65.2			
	86.0		75.1		13.4		49.6		19.7		72.6			
FAS (10 mg/kg) + AO (5 mL/kg)	19.8	14.3 ± 3.4 **	14.7	12.3 ± 2.4 **	3.8	4.4 ± 2.0	19.1	23.7 ± 3.7	6.3	5.6 ± 0.9 **	16.0	10.5 ± 4.5	ND	ND
	15.7		14.0		4.2		26.3		5.2		11.5			
	16.9		15.1				28.3		4.1		16.9			
	10.9		9.8		3.5		19.0		5.1		7.4			
	11.7		10.9		3.0		25.6		6.1		8.7			
	14.1		12.2		8.5		25.5		5.5		5.7			
	10.7		9.2		3.5		22.3		6.6		7.3			
COUM (60 mg/kg) + FAS (10 mg/kg)	17.4	14.2 ± 1.9 **,##	15.6	12.8 ± 1.6 **,##	4.9	3.3 ± 0.9 ##	28.4	22.2 ± 3.6 ##	5.7	5.6 ± 0.6 *,##	12.5	11.4 ± 2.3	ND	ND
	14.7		12.7		3.3		22.5		6.6		11.4			
	14.1		12.9		2.9		23.3		5.1		11.2			
	13.4		12.5		2.5		23.0		5.0		10.9			
	15.6		14.0				18.1		6.2		15.6			
	13.0		11.3		2.7		17.9		5.4		10.3			
	11.5		10.9		3.5		22.4		5.3		8.0			
Experiment 2														
AO (5 mL/kg)	34.1	25.3 ± 6.2	29.0	22.1 ± 5.4	6.8	5.4 ± 1.1	26.9	33.6 ± 7.3	9.9	10.0 ± 1.4	27.3	19.9 ± 5.1	277.8	299.6 ± 37.5
	22.6		19.5		4.6		45.2		9.4		18.0		321.5	
	33.8		30.0		6.9		42.2		12.3		26.9		309.1	
	20.4		17.6		4.7		31.8		8.7		15.7		262.1	
	23.2		20.4		5.2		33.1		9.1		18.0		252.9	
	24.2		22.3		5.4		28.9		11.4		18.8		359.7	
	18.8		16.0		4.1		27.4		9.0		14.7		313.9	
E_2B (400 µg/kg)	123.4	126.3 ± 29.0 **	94.3	91.2 ± 11.0 **,#	19.4	17.9 ± 2.3 **	75.9	64.4 ± 8.6 **	29.7	28.5 ± 2.7 **	104.0	108.4 ± 27.3 **	452.5	447.5 ± 129.0 **
	126.1		86.6		17.4		66.1		32.0		108.7		524.6	
	115.3		91.5		18.4		69.9		25.7		96.9		527.2	
	163.8		109.1		21.0		70.3		30.7		142.8		285.2	
	164.8		99.3		18.9		54.9		29.7		145.9		635.8	
	102.1		77.2		14.1		61.4		24.9		88.0		417.5	
	88.3		80.6		15.9		52.5		27.1		72.4		289.8	
E_2B (80 µg/kg)	149.6	132.6 ± 17.8 **,##	97.6	94.7 ± 8.4 **,##	19.4	17.8 ± 1.4 **	75.2	64.6 ± 10.7 **	29.4	27.2 ± 4.3 **	130.2	114.2 ± 18.2 **,##	521.5	534.7 ± 45.6 **
	125.6		89.9		17.0		56.6		28.9		108.6		443.8	
	134.5		102.0		18.0		53.8		21.8		116.6		543.1	
	157.1		104.0		19.3		82.4		33.1		137.8		578.8	
	136.0		100.7				65.8		21.3				540.1	
	121.3		83.9		16.2		61.0		26.7		105.1		579.6	
	103.8		84.5		16.6		57.1		29.4		87.2		536.2	
COUM (60 mg/kg)	106.3	100.3 ± 12.5 **	92.7	79.6 ± 8.0 **	19.5	15.9 ± 1.8 **	66.6	69.8 ± 12.9 **	27.5	28.8 ± 4.4 **	86.8	84.3 ± 11.7 **	647.0	504.7 ± 200 *
	111.6		85.9		16.9		76.3		38.1		94.7			
	103.6		79.7		15.9		81.1		29.6		87.7		277.9	
	112.4		78.4		14.6		54.7		26.1		97.8		760.5	
	77.9		69.2		14.7		89.9		28.8		63.2		354.1	
	100.2		79.9		15.9		59.4		24.9		84.3			
	89.9		71.4		14.1		60.9		26.6		75.8		484.1	

Abbreviations: AO, arachis oil; COUM, coumestrol; E_2B, estradiol benzoate; FAS, Faslodex (ICI 182,780; Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK); Ind, individual; ND, not determined; SD, standard deviation; ULF, uterine luminal fluid. The entire reproductive tract was removed from the animal, thus allowing the determination of vaginal, cervical, and uterine weights for each animal. Uterine weight was determined preblotting (intact uterus) and postblotting (blotted uterus) of excess fluid. Total uterine dry weight was also calculated for each animal based on the right uterine horn dry weight. The left uterine horn plus its junction with the cervix was used either to determine uterine hyperplasia (experiment 1; Table 2) or DNA content of the uterus (experiment 2). Data were assessed for statistical significance using analysis of variance (*10*).

[a]Not included in statistical analyses. *$p < 0.05$ (vs. controls). **$p < 0.01$ (vs. controls). #$p < 0.05$ (vs. COUM). ##$p < 0.01$ (vs. COUM).

Determination of uterine hyperplasia. The left uterine horn together with the junction to the cervix from each animal was fixed in 10% formal saline and processed to paraffin wax. Longitudinal sections were stained to reveal BrdU (*7*) as follows. The sections were trypsinized, the DNA denatured, and the endogenous peroxidase activity blocked. The sections were then incubated with normal goat serum followed by anti-BrdU antibody, washed, incubated with goat antimouse immunoglobulins, washed, and incubated with Strep A B complex/horseradish peroxidase. The sections were developed with 3,3-diaminobenzidene and counter-stained with Mayer's hematoxylin. The percentage labeling index was determined for epithelial cells of the uterine lumen and glands and for the stroma (500 cells per animal assessed where possible). Endometrial and epithelial thickness were assessed at 10 locations for each animal.

Determination of uterine DNA content. The left uterine horn and its junction to the cervix from each animal was homogenized for 10–15 sec in 1 mL chilled physiological saline using an Ultra-Turrax (Janke and Kunkel IKA-Labortechnik, Stauffen, Germany). Hydrolyzed uterine total nucleic acid was isolated as follows using trichloroacetic acid (TCA) (*8*). Chilled 10% TCA (0.5 mL) was added to an equal volume of homogenate, vortexed, and incubated on ice for 30 min. This mixture was spun at 600*g* at 4°C for 5 min, the supernatant carefully removed, and the pellet was washed twice using 1 mL chilled 5% TCA. The nucleic acid was hydrolyzed in a further 1 mL 5% TCA at 90°C for 30 min. This mixture was cooled to 4°C on ice before centrifuging at 600*g* at 4°C for 15 min. Duplicate dilutions of each supernatant (1:30 in 5% TCA) were prepared and were allowed to reach room temperature before determining their hydrolyzed nucleic acid content using an optical density (OD) of 260 nm. Known quantities of calf thymus DNA were handled in an identical manner (with the exception of the homogenization step) so that a calibration curve could be generated. From this standard curve the amount of nucleic acid (in micrograms) in each sample could be calculated and hence, the amount of nucleic acid/mg of tissue could be determined. Assuming even distribution of nucleic acid throughout the entire uterus, the amount of nucleic acid (micrograms) per uterus was then calculated.

Total uterine RNA content was determined for each tissue sample using the 5% TCA hydrolysates prepared as described above (*8*). Briefly, 250 mL hydrolysate was mixed with an equal volume of 5% TCA and 1 mL orcinol reagent (*9*). The mixture was heated to 100°C for 15 min and cooled to room temperature before determining the OD at 660 nM. Yeast RNA standards were prepared using the same method as for the uterine tissue samples (after homogenization) to generate a standard calibration curve. From this curve the RNA content (in micrograms) of the uterine horn, and hence the entire uterus, could be estimated. Total DNA content was determined by subtracting this RNA value from the total nucleic acid estimation as described above (experiment 2, Table 1).

Statistical assessment of data. All data in Tables 1 and 2 were assessed for statistically significant differences by analysis of variance (*10*). Differences between the groups in their labeling indices (hyperplasia; Table 3) were determined by analysis of variance following a double arcsine transformation (*11*). Comparisons were carried out separately for all observations between the control and all test groups as well as between COUM versus E_2B and, where appropriate, between COUM versus COUM + FAS.

Table 2. Hypertrophy of the endometrium and luminal epithelium in the immature rat following treatment with E_2B, COUM, or FAS.

Test agent and daily dose level	Endometrium[a]	Epithelium[a]
AO (5 mL/kg)	126.4 ± 23.6	8.5 ± 1.3
E_2B (400 µg/kg)	225.6 ± 42.8**	42.8 ± 9.8**,##
COUM (60 mg/kg)	231.3 ± 45.7**	22.1 ± 4.7**
COUM (60 mg/kg) + FAS (10 mg/kg)	120.5 ± 16.6##	8.7 ± 1.8##
FAS (10 mg/kg)	100.4 ± 25.5	9.2 ± 2.0

Abbreviations: AO, arachis oil; COUM, coumestrol; E_2B, estradiol benzoate; FAS, Faslodex (ICI 182,780; Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK).
[a]Height (µm) mean ± standard deviation (n = 7). Data were assessed for statistical significance by analysis of variance (*10*). **p < 0.01 (vs. control). ##p < 0.01 (vs. COUM).

Results

Uterotrophic assays. The results of the two experiments conducted are shown in Table 1; experiment 1 is also shown in Figure 1. In both experiments E_2B gave the expected maximal uterotrophic response at the higher dose evaluated, and also at the lower dose used in the second experiment. COUM gave a positive uterotrophic response of a magnitude similar to that reported earlier (*4*). In addition, increases in the weights of the vagina and cervix and in total uterine luminal fluid content were also seen for both chemicals in both experiments (Table 1). Coadministration of FAS abolished the uterotrophic response given by COUM, and FAS itself led to a significant reduction in uterine weights, as previously reported (*4*). A proportion of the animals treated with E_2B presented with open vaginas at termination

Table 3. Labeling indices in the immature rat endometrium following treatment with E_2B, COUM, or FAS.

Test agent and daily dose level	Uterine lumen[a]	Glandular epithelium[a]	Stroma[a]
AO (5 mL/kg)	1.6 ± 0.6 (n = 6)	4.1 ± 1.3 (n = 6)	5.6 ± 3.3
E_2B (400 µg/kg)	22.7 ± 13.3**,##	42.3 ± 14.3**,##	12.2 ± 6.9*
COUM (60 mg/kg)	62.0 ± 8.3 (n = 6)**	70.0 ± 7.6**	11.1 ± 3.8**
COUM (60 mg/kg) + FAS (10 mg/kg)	2.7 ± 2.9##	2.9 ± 5.0##	0.7 ± 0.3**,##
FAS (10 mg/kg)	1.2 ± 0.5	1.3 ± 1.5*	0.8 ± 0.2*

Abbreviations: AO, arachis oil; COUM, coumestrol; E_2B, estradiol benzoate; FAS, Faslodex (ICI 182,780; Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK).
[a]Labeling index (%) mean ± standard deviation (n = 7, except where indicated). Data were assessed for statistical significance by analysis of variance following a double arcsine transformation (*11*). *p < 0.05 (vs. controls); **p < 0.01 (vs. controls); ##p < 0.01 (vs. COUM).

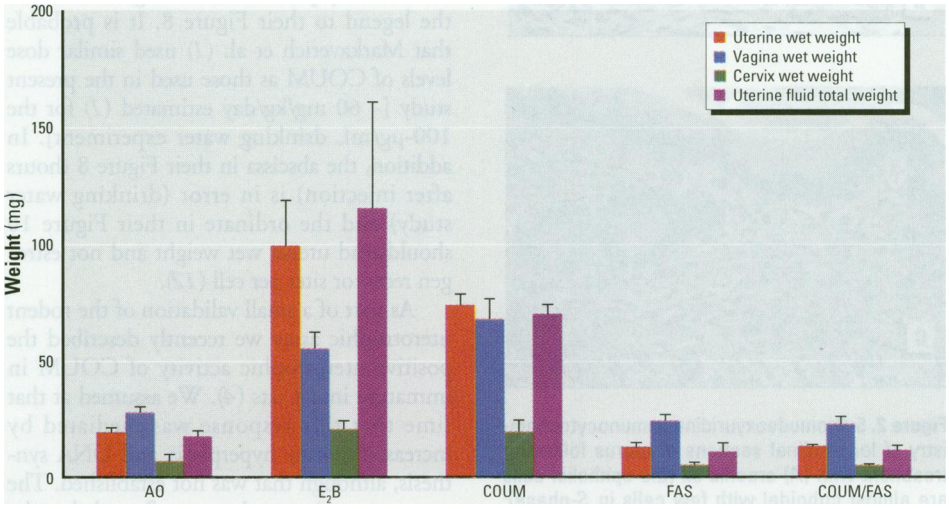

Figure 1. Activity of E_2B, COUM, and FAS in the immature rat uterotrophic assay. Abbreviations: AO, arachis oil; COUM, coumestrol; E_2B, estradiol benzoate; FAS, Faslodex (ICI 182,780; Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK). AO was the common vehicle. Data were taken from experiment 1, Table 1. The total weight of uterine fluid was also calculated. Data are shown as mean weights ± standard deviation.

(Table 1), but all AO-, COUM-, and FAS-dosed animals had closed vaginas.

Determination of uterine hyperplasia. Animals treated with E_2B (experiment 1) had significantly increased levels of BrdU staining in the endometrium, particularly in the luminal and glandular epithelium ($p < 0.01$ vs. controls in both tissues; Table 3 and Figure 2). Animals treated with COUM showed similar increases in BrdU incorporation to those seen with E_2B ($p < 0.01$ vs. controls; Table 3 and Figure 2). Coadministration of COUM and FAS inhibited hyperplasia. Uterine epithelial and endometrial height paralleled hyperplasia (Table 2).

Determination of uterine DNA content. Total DNA content of the uteri from animals in experiment 2 are shown in Table 1. Animals treated with COUM showed the same level of increase in DNA content as the animals exposed to the two dose levels of E_2B. The average DNA content of control animal uteri (299.6 ± 37.5 µg/total uterus) is similar to that reported by Markaverich et al. (*1*): 217 µg/uterus, as stated in the legend to their Figure 8.

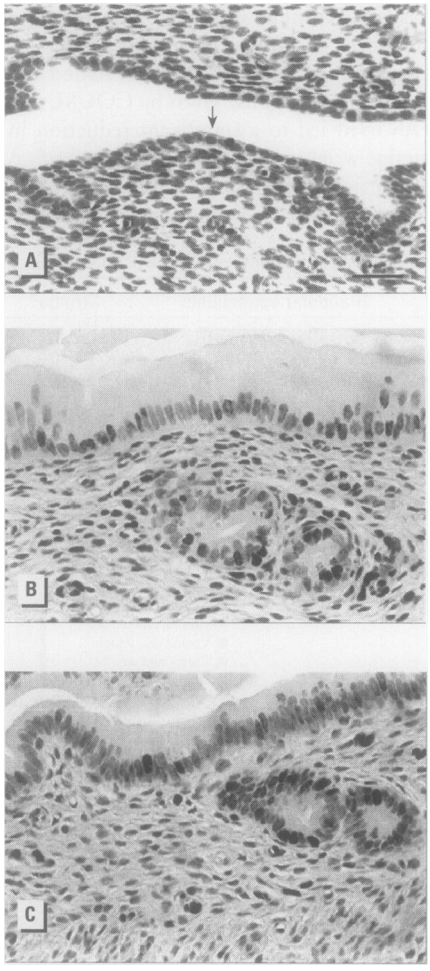

Figure 2. 5-Bromodeoxyuridine immunocytochemistry of longitudinal sections of uterus following treatment with (*A*) arachis oil (the epithelial cells are almost cuboidal with few cells in *S*-phase); (*B*) estradiol benzoate (400 µg/kg; columnar epithelium with many cells in *S*-phase); or (*C*) coumestrol (60 mg/kg; narrow columnar epithelial cells with many cells in *S*-phase). Bar = 50 µm.

Discussion

The rodent uterotrophic assay is widely and increasingly used as a primary assay for estrogenic activity. Investigators rely primarily on either the rat or the mouse, and on either immature intact animals or ovariectomized animals. Data derived from assays using any of these protocol variants are generally considered qualitatively comparable. Underlying this belief is the assumption that increases in uterine weight are always driven by increased uterine hyperplasia in concert with increased uterine fluid accumulation (imbibation). Within this context, the report by Markaverich et al. (*1*) that the uterotrophic activity of COUM in the ovariectomized rat assay was not associated with an increased DNA content in the uterus was of obvious interest. Thus, there may be two distinct mechanisms for the production of a positive uterotrophic assay response—a genuine hyperplastic response and an atypical response driven by increased cellular hypertrophy associated only with increased water imbibation and protein production. Markaverich et al. (*1*) stated that their findings were in contrast to the reports of others (*2–4*) concerning the uterotrophic activity of COUM in immature (intact) rats, although they also noted that uterine hyperplasia and DNA contents had not been determined in those earlier experiments. Comparisons between the present data and those of Markaverich et al. (*1*) are difficult because of the general absence of primary data in the Markaverich et al. paper. In particular, the critically important uterine DNA content data are only presented as a single number in the legend to their Figure 8. It is probable that Markaverich et al. (*1*) used similar dose levels of COUM as those used in the present study [~ 60 mg/kg/day estimated (*1*) for the 100-µg/mL drinking water experiment]. In addition, the abscissa in their Figure 8 (hours after injection) is in error (drinking water study) and the ordinate in their Figure 10 should read uterus wet weight and not estrogen receptor sites per cell (*12*).

As part of a small validation of the rodent uterotrophic assay we recently described the positive uterotrophic activity of COUM in immature intact rats (*4*). We assumed at that time that this response was mediated by increased uterine hyperplasia and DNA synthesis, although that was not established. The present experiments have confirmed that the trophic activity of COUM to the intact immature rat is evident for both wet and dry uterine weights and for wet weights of the cervix and vagina. Further, uterine fluid imbibation is increased, the effects are mediated via the estrogen receptor, and they are accompanied by increases in uterine hyperplasia and total uterine DNA content. In fact, the uterotrophic response elicited by COUM was identical to that induced by E_2B in all of these respects.

These data therefore indicate a possible difference between the immature intact rat uterotrophic response to COUM and the ovariectomized rat uterotrophic response to COUM. However, before that conclusion can be drawn with any confidence it will be necessary for the original observations reported by Markaverich et al. (*1*) to be confirmed and extended. This is necessary because those observations were limited in respect to the critical absence of a hyperplastic response of the uterus to COUM. In particular, uterine hyperplasia was neither assessed histopathologically nor with the aid of a marker of cell division (such as BrdU incorporation). Rather, reference was made only to a single uterine DNA content figure, and then only in the legend to one of the figures in the paper [Figure 8; (*1*)]. Such a repeat experiment was not conducted as part of the present studies because of the scarcity and expense of COUM, especially given the larger quantities that would be required for the conduct of mature ovariectomized rat uterotrophic assays.

References and Notes

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